

Inhibition of Wound-Induced Accumulation of Allene Oxide Synthase Transcripts in Flax Leaves by Aspirin and Salicylic Acid¹

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Allene oxide synthase (AOS) mediates the conversion of lipoxygenase-derived fatty acid hydroperoxides to unstable allene epoxides, which supply the precursors for the synthesis of the phytohormone jasmonic acid (JA). In this study the characterization of AOS gene expression in flax (*Linum usitatissimum*) is reported. AOS was constitutively expressed in different organs of flax plants. Additionally, AOS gene expression was enhanced after mechanical wounding in both the directly damaged leaves and in the systemic tissue located distal to the treated leaves. This wound-induced accumulation of AOS required the de novo biosynthesis of other unknown proteins involved in the signaling pathway modulating wound-induced AOS gene expression. Furthermore, the wound-induced AOS mRNA accumulation was correlated with the increase in the levels of JA. Both JA and its precursor, 12-oxo-phytodienoic acid, activated AOS gene expression in a dose-dependent manner. Thus, JA could activate its own biosynthetic pathway in flax leaves. Moreover, neither salicylic acid (SA) nor aspirin influenced AOS enzymatic activity. It is interesting that pretreatment with SA or aspirin inhibited wound-induced accumulation of AOS transcripts. These results suggest that a potent inhibition of JA biosynthetic capacity in leaves can be affected by SA or aspirin at the level of AOS gene expression.

JA and related compounds, such as its methyl ester (methyl jasmonate) and certain amino acid conjugates, are ubiquitous cyclopentanone compounds that have been implicated in several physiological processes in plant metabolism (Sembdner and Parthier, 1993; Farmer, 1994). Several studies have been reported suggesting a role for these substances in a signal transduction pathway involved in the expression of plant defense genes in response to various stresses (Farmer and Ryan, 1992; Sembdner and Parthier, 1993; Peña-Cortés et al., 1995).

AOS (EC 4.2.1.92, hydroperoxide dehydratase) is an enzyme involved in the biosynthetic pathway of JA (Vick and Zimmerman, 1987; Hamberg and Gardner, 1992). In the principal sequence of reactions, linolenic acid is oxygenated by a lipoxygenase, and the resulting 13-hydroperoxide is converted to an allene oxide by the AOS. This enzyme

converts fatty acid hydroperoxides to unstable allene epoxides (Song and Brash, 1991), which either spontaneously or through the action of an AOC (Hamberg and Fahlstadius, 1990) cyclize to form cyclopentanone acids, thereby providing the direct precursor of JA. The ability of AOS to rapidly metabolize hydroperoxylinolenic acid into α - and γ -ketol fatty acids implicates this type of oxygenase in the biosynthetic pathway of the phytohormone JA.

AOS from flax (*Linum usitatissimum*) plants has been characterized (Vick and Zimmerman, 1987; Brash and Song, 1995), isolated and cloned (Song and Brash, 1991; Song et al., 1993), and classified as a novel class of Cyt P450 enzymes now defined as CYP74. Pan et al. (1995) reported the identification of the major protein of guayule rubber particles as a CYP74, which shows 85% similarity to the flax enzyme. Recently, Laudert et al. (1996) cloned a cDNA encoding an AOS from *Arabidopsis*. Like flax AOS, the *Arabidopsis* protein contains a signal peptide that has the features of a chloroplast transit peptide (Laudert et al., 1996).

Increased levels of JA have been observed after desiccation treatment of leaf segments (Reinbothe et al., 1994) and after mechanical wounding in soybean (Creelman et al., 1992), tomato (Peña-Cortés et al., 1993), and potato plants (Peña-Cortés et al., 1995). However, this wound-induced accumulation of JA and the subsequent activation of gene expression can be prevented by SA or aspirin (Doherty et al., 1988; Peña-Cortés et al., 1993; Doares et al., 1995). Previous results have shown that SA or aspirin inhibit the conversion of 13-HPLA to 12-oxo-PDA, thereby inhibiting the signal pathway by blocking the synthesis of JA (Peña-Cortés et al., 1993). Doares et al. (1995) reported that the inhibition of synthesis of proteinase inhibitor proteins and mRNAs by SA in both light and darkness also occurs at a step in the signal transduction pathway after JA biosynthesis, but preceding transcription of the inhibitor genes. Recently, we demonstrated the involvement of the flax AOS in the biosynthesis of JA (Harms et al., 1995). Transgenic potato plants overexpressing the flax AOS cDNA exhibited increased endogenous levels in JA. Furthermore, the flax-

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Abbreviations: AOC, allene oxide cyclase; AOS, allene oxide synthase; 13-HPLA, 13-hydroperoxylinolenic acid; JA, jasmonic acid; NF- κ B, nuclear factor κ B; 12-oxo-PDA, 12-oxo-phytodienoic acid; SA, salicylic acid.

derived AOS protein accumulated in the chloroplast of the transgenic plants. These results showed that the JA biosynthesis, or at least a part of it, occurs in the chloroplast (Harms et al., 1995).

Recently, Laudert et al. (1996) reported evidence of AOS gene expression and its relation to the accumulation of JA in *Arabidopsis* plants. To further examine the mechanism involved in the regulation of AOS gene expression and to gain a broader insight into the role of JA on its own biosynthetic pathway, we analyzed AOS gene expression in flax plants. AOS is constitutively expressed in different organs. Additionally, AOS gene expression can be enhanced in flax leaves after mechanical wounding. Wound-induced AOS gene activation is not restricted to the directly damaged tissue but also takes place in the leaves located distal (systemic tissue) to the wounded leaves. The wound-induced AOS gene can be blocked by cycloheximide, suggesting the requirement of de novo protein biosynthesis. Exogenous application of JA precursor 12-oxo-PDA or JA to the flax leaves leads to an accumulation of AOS mRNAs in a dose-dependent manner. It is interesting that wound-induced AOS gene expression correlates with the increase of endogenous levels of JA. This increase can be inhibited by aspirin or SA. Neither substance influences AOS enzymatic activity but, rather, alter the wound-induced AOS gene expression.

MATERIALS AND METHODS

Plant Material and Chemical Substances

Flax (*Linum usitatissimum*) plants were grown under greenhouse conditions (26°C day/20°C night, 70%–80% RH, and 14 h of light). Linolenic acid, aspirin, and SA were obtained from Sigma. (–)-JA was purchased from Apex Organics (Leicestershire, UK).

Treatment of Flax Plants

Four- to six-week-old flax plants (30–40 cm high) were cut at the base of the stem and placed in a solution containing water alone or water supplemented with the following compounds: cycloheximide, chloramphenicol, SA, aspirin, or JA. For wounding of the plant, flax leaves were put on a stable base and the ribbed end of a pen was rolled once over its surface parallel to the mid vein. Systemic leaves correspond to those located above the wounded ones. Samples were collected at different times after treatment or wounding, frozen immediately in liquid nitrogen, and stored at –80°C until further analysis.

RNA Gel-Blot Analysis

For RNA isolation leaf material was immediately frozen after harvesting in liquid nitrogen and kept at –80°C until processing. Total RNA was extracted from the frozen leaf material according to the method of Logemann et al. (1987). Blotting and hybridization conditions were as described by Amasino (1986). Probes used for radioactive labeling were an AOS cDNA fragment (Song et al., 1993) and potato 28S

rRNA cDNA (data not shown). The 28S rRNA gene was used as a loading control. Each experiment was independently repeated at least three times. The film was exposed overnight for 14 h at –80°C. The signals on the x-ray films were quantified densitometrically with a densitometer (model GS-670, Bio-Rad), and the AOS transcript level was plotted as a percentage of maximum transcript level. The northern blots and JA quantitations shown in the various figures are representatives of the average situation.

Extraction and Analysis of Jasmonate

Frozen leaf samples stored at –80°C were homogenized in 80% methanol, essentially as described (Kogel et al., 1995). After removal of the insoluble material by centrifugation, supernatants were passed through a Sep-Pak C₁₈ cartridge (Merck, Darmstadt, Germany) to remove chlorophyll and other lipophilic compounds. After evaporation the aqueous phase was adjusted to 10 mL with a solution of 30% (v/v) methanol and 1% acetic acid, and added onto a C₁₈ column. The jasmonate was eluted with 80% acidic methanol. The eluate was concentrated and used for JA determination by ELISA according to the method of Knöfel et al. (1990).

Assay of AOS Activity

AOS enzymatic activity was assayed by formation of 12-oxo-PDA in 50 mM sodium phosphate buffer, pH 6.3, containing 0.1 mM 13-HPLA as the substrate. The reaction was started by the addition of 0.1 mL of crude leaf extract (1 mg/mL total protein). After an incubation time of 90 s at 25°C, the mixture was acidified with 0.1 M HCl to pH 2.8. The measurement of 12-oxo-PDA was as described by Harms et al. (1995).

RESULTS

AOS Gene Expression in Flax Plants

The enzyme AOS is ubiquitous in plants (Brash and Song, 1995; Simpson and Gardner, 1995) and is part of a metabolic pathway that converts linolenic acid to JA, a phytohormone and signaling molecule in plant defense (Farmer and Ryan, 1992). Overexpression of the flax cDNA encoding AOS in potato plants led to an increase of the endogenous levels of JA, which were 6- to 12-fold higher than those present in normal plants. The flax AOS protein was found to accumulate in the chloroplasts of the transgenic potato lines (Harms et al., 1995), suggesting that the biosynthesis of JA, or at least a part of it, takes place in this compartment. To gain a broader insight and to define the spatial expression pattern of AOS during normal plant development, RNAs from different tissues of flax plants were isolated and analyzed by the northern-blot technique. The presence of AOS mRNA was detected by hybridizing the blots with a radioactively labeled probe of flax AOS cDNA (Song et al., 1993). The amount of RNA on the membranes in this and subsequent experiments was checked by hybridizing them to a 28S rRNA probe (data

not shown). AOS transcripts were detected in all of the organs analyzed (Fig. 1). AOS mRNA was present in seeds, roots, stems, old and young leaves, and floral buds. Although no significant differences were observed in the content of AOS transcripts in most of the organs, the flax seeds showed a slight increase in levels of AOS mRNA.

Mechanical Wounding Induces AOS Gene Expression in a Local and Systemic Manner

JA accumulation is induced by mechanical wounding in different plant species (Creelman et al., 1992; Albrecht et al., 1993; Baldwin et al., 1994; Bleichert et al., 1995; Doares et al., 1995; Peña-Cortés and Willmitzer, 1995). Because AOS is involved in the formation of allene oxides, the precursor of JA, we decided to analyze the effect of mechanical damage on AOS gene expression. To this end, flax leaves were mechanically wounded as described in "Materials and Methods." Directly damaged and systemic unwounded leaves were harvested at different times, and total RNA was extracted for analysis of AOS gene expression by northern-blot analysis. AOS mRNA started to accumulate as soon as 1 h after wounding (Fig. 2). The highest accumulation of AOS mRNA was observed 6 h after treatment; thereafter, levels declined and after 26 h showed levels similar to those observed in the control plants before treatment. AOS mRNA accumulation also

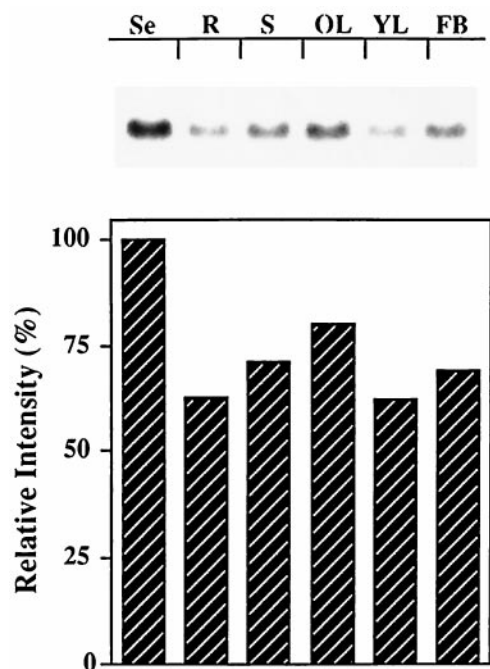


Figure 1. AOS gene expression in flax plants. RNA was isolated from intact flax organs such as seeds (Se), roots (R), stems (S), old leaves (OL), young leaves (YL), and floral buds (FB), and subjected to RNA gel-blot analysis. Blots were probed with the flax AOS cDNA. Equal amounts of RNA were loaded, as confirmed by staining with ethidium bromide and hybridization with a rRNA probe (data not shown). The autoradiogram and the corresponding densitogram are shown. Data are from one of three separate experiments all showing the same results.

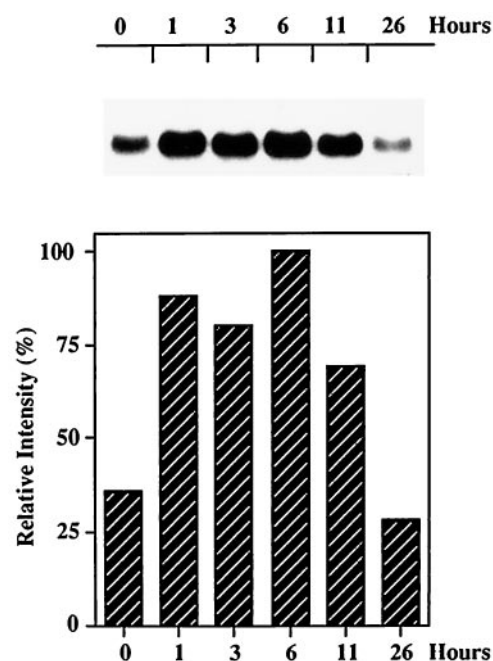


Figure 2. Induction of AOS upon mechanical wounding in leaves of flax plants. Total RNA was isolated from flax leaves at the times shown after mechanical injury and then subjected to gel-blot analysis. Amounts of RNA were confirmed as described in Figure 1. Blots were probed with the flax AOS cDNA and the corresponding densitogram was quantified densitometrically as described in Figure 1.

occurred in the systemic unwounded leaves of the wounded plants (Fig. 3). As in wounded leaves, the accumulation of AOS mRNA in the systemic unwounded leaves took place in the first 6 h, the levels returning to those of the control plants 20 h after wounding. These results demonstrate that mechanical wounding leads to a local and systemic activation of AOS gene expression, as detected by steady-state RNA amounts.

Wound-Induced Accumulation of AOS mRNA Requires Protein Synthesis

Our initial studies of the molecular mechanism of wounding action investigated whether protein synthesis was required for AOS gene expression. Detached leaves were supplied in the light with either 300 μ M chloramphenicol (an inhibitor of protein synthesis acting in the chloroplast) or 30 μ M cycloheximide (a cytoplasmic protein-synthesis inhibitor). The inhibitors had opposite effects on the wound-induced accumulation of AOS mRNA (Fig. 4). Cycloheximide treatment clearly blocked the accumulation of AOS mRNA after wounding, whereas chloramphenicol did not. These data suggest that wound-induced AOS gene expression requires cytoplasmic protein synthesis and that the factors mediating the induction of these genes are newly synthesized upon wounding.

AOS Gene Expression Is Activated by JA

Exogenous application of JA (Creelman et al., 1992; Farmer and Ryan, 1992; Gundlach et al., 1992; Peña-Cortés

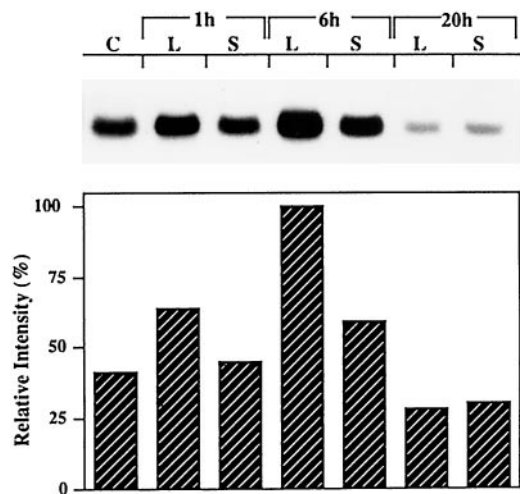


Figure 3. Local and systemic accumulation of AOS mRNA upon mechanical wounding in flax plants. RNA was isolated from flax leaves of intact plants before wounding (C) and from directly wounded (L) and systemic unwounded (S) leaves at the times shown after treatment. Amounts of RNA were confirmed as described in Figure 1. Blots were probed with the flax AOS cDNA and the corresponding densitogram is shown.

et al., 1993) and components of the JA biosynthetic pathway (Farmer and Ryan, 1992; Peña-Cortés et al., 1993, 1995) initiates the accumulation of several mRNAs from genes involved in different physiological plant processes (Sembdner and Parthier, 1993; Farmer, 1994). For instance, JA spraying of tomato or potato plants leads to activation of the expression of the wound-inducible *pin2* genes. This accumulation is also seen upon the exogenous application of different components of the JA biosynthetic pathway, such as linolenic acid and 12-oxo-PDA (Farmer and Ryan, 1992; Peña-Cortés and Willmitzer, 1995). To determine whether JA affects AOS gene expression, detached flax leaves were supplied with a solution containing 10 and 50 μM JA. As shown in Figure 5, JA initiated the accumulation of AOS mRNA in a dose-dependent manner. Furthermore, lower concentrations of JA (10 μM) led to a delayed accumulation of AOS mRNA compared with 50 μM . Similar results were obtained by using concentrations lower or higher than 10 or 50 μM JA and 12-oxo-PDA (data not shown). The results suggest that some precursors of the JA biosynthetic pathway and JA itself are able to activate their own synthesis in flax leaves.

Wound-Induced Accumulation of JA Is Blocked by Aspirin

Because mechanical wounding initiates the accumulation of JA in different plant species (Creelman et al., 1992; Albrecht et al., 1993; Peña-Cortés et al., 1993, 1995; Reinbothe et al., 1994), we decided to determine the changes in the endogenous levels of JA in flax plants after mechanical damage. JA levels increased within 1 h (from 460 to 5400 pmol JA equivalents g^{-1} fresh weight) and reached a maximum (6700 pmol JA equivalents g^{-1} fresh weight) 6 h after wounding. Thereafter, levels of JA declined but remained

clearly elevated until 26 h after wounding (4200 pmol JA equivalents g^{-1} fresh weight).

To test the inhibitory effect of SA or aspirin on the wound-induced JA accumulation previously observed in tomato plants (Peña-Cortés et al., 1993; Doares et al., 1995), detached flax leaves were pretreated for 3 h with either 1 mM aspirin or 1 mM SA and subsequently wounded. At different times after the treatment, the directly wounded leaves (local leaves) were harvested and JA levels were determined. As in tomato, flax plants pretreated with aspirin showed similar levels of JA as the unwounded control plants (Fig. 6). The same results were obtained when flax plants were pretreated with 1 mM SA (data not shown). These results indicate that both substances block the wound-induced JA accumulation and confirm previous observations in tomato plants (Peña-Cortés et al., 1993; Doares et al., 1995).

Aspirin Effect on AOS Activity

SA and aspirin have been implicated in several biological processes in different organisms. For instance, in plants both substances are able to increase resistance against certain pathogens (Vernooij et al., 1994). Doherty et al. (1988) demonstrated that aspirin and derivatives of SA block the wound-induced accumulation of *pin2* protein. Both substances also repress the wound-induced accumulation of *pin2* mRNA by blocking the biosynthesis of JA (Peña-Cortés et al., 1993). Furthermore, both 12-oxo-PDA and JA overcome the inhibitory effect of aspirin or SA, whereas

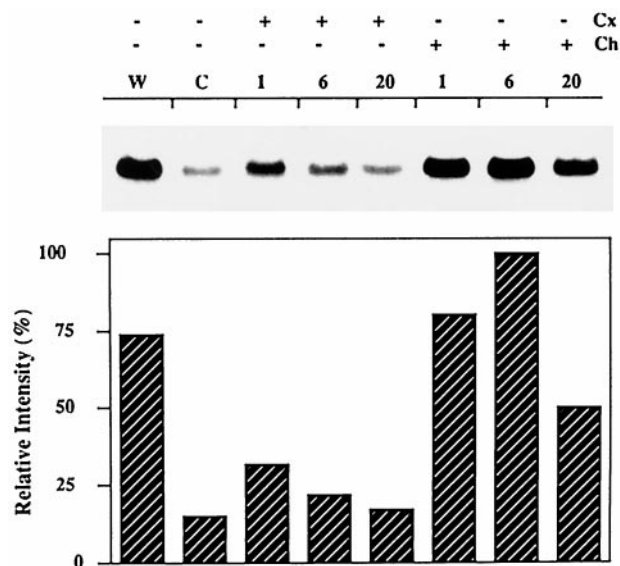


Figure 4. The effects of cycloheximide (Cx) and chloramphenicol (Ch) on wound-induced AOS mRNA accumulation. Flax plants were preincubated for 2 h with cycloheximide or chloramphenicol as described previously and were subsequently wounded. RNA was isolated from the flax leaves at the times shown after treatment, with abbreviations as given in Figure 3. The AOS mRNA levels are compared with levels in nontreated leaves (C) and with AOS mRNA levels in wounded flax leaves (W) 6 h after treatment in the absence of cycloheximide and chloramphenicol.

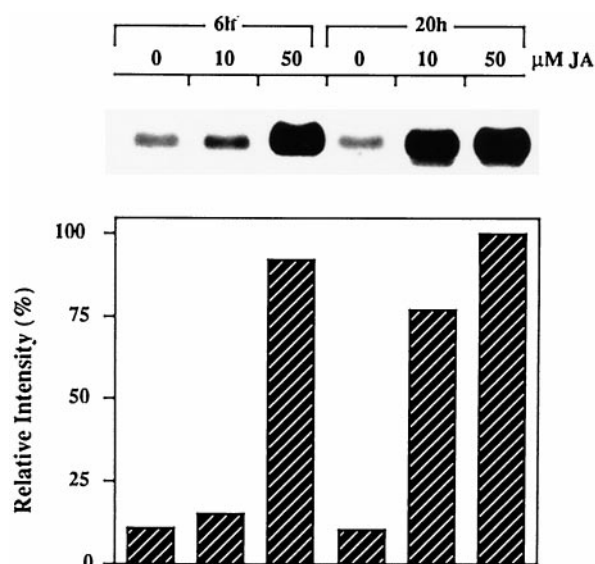


Figure 5. Effect of JA on AOS gene expression. RNA was isolated from detached flax plants cut at the base of the stem and supplied with buffer alone (0), 10 μM JA (10), or 50 μM JA (50) and subjected to RNA gel-blot analysis. The blot was probed as indicated and the corresponding densitogram is shown.

linolenic acid and 13-HPLA do not. Thus, it was postulated that aspirin and SA block the wound-induced JA accumulation by inhibiting AOS activity (Peña-Cortés et al., 1993).

To determine whether SA or aspirin affect the enzymatic activity of AOS, its capacity for conversion of 13-HPLA to 12-oxo-PDA was examined in the presence and absence of these substances. To this end, total protein from flax seed was isolated (Song and Brash, 1991) and incubated in buffer either with or without different concentrations of SA and aspirin. None of the concentrations of SA or aspirin used showed any effect on AOS activity (Fig. 7). Even at higher concentrations (up to 3 mM) the ability of the enzyme to form 12-oxo-PDA was not affected (data not

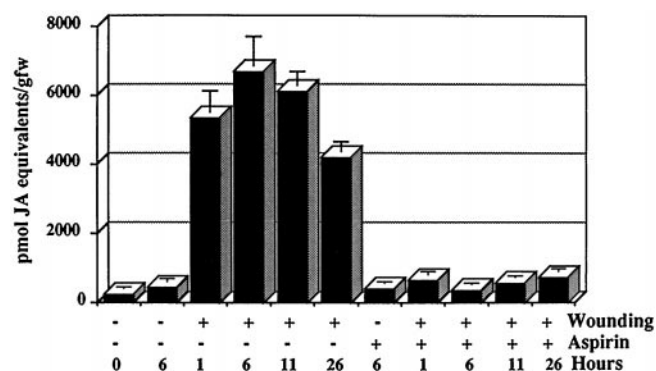


Figure 6. Effect of aspirin on wound-induced accumulation of JA in flax leaves. Flax plants were preincubated for 2 h with buffer or aspirin, as described previously, and were subsequently wounded. RNA was isolated from the flax leaves at the times shown after treatment of the excised plants, as given in Figure 3. The levels of endogenous JA in wounded plants were compared in the presence and absence of aspirin. Values are the average of three samples in three independent experiments. gfw, Grams fresh weight.

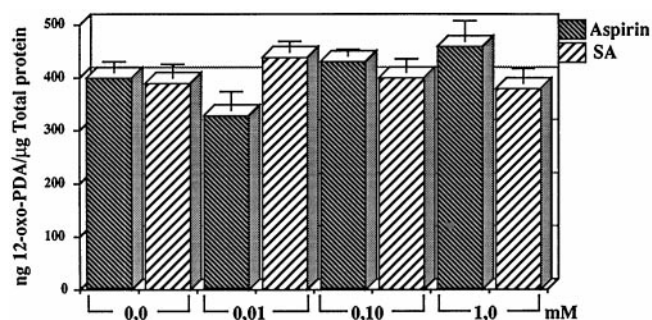


Figure 7. Effect of aspirin and SA on AOS activity. The enzymatic activity of AOS was analyzed as described by Harms et al. (1995) in the absence and presence of increasing concentrations of either aspirin or SA. Specific AOS activity in flax leaf extracts is given in nanograms of 12-oxo-PDA per microgram of total protein.

shown). These data suggest that SA and aspirin do not inhibit the enzymatic activity of flax AOS.

Wound-Induced AOS Gene Expression Is Blocked by Aspirin

Because wound-induced JA accumulation is inhibited by SA and aspirin and neither substance affected AOS enzymatic activity, we decided to analyze the effect of these substances on the wound-induced AOS gene expression at the RNA level. To this end, detached flax leaves were incubated in water or pretreated with aspirin or SA and subsequently wounded. Because wound-induced AOS gene expression reaches its maximum 6 h after wounding (Fig. 2), total RNA was isolated from leaves 6 and 20 h after treatment. As expected, mechanical wounding led to an accumulation of AOS in both the directly wounded and the systemic unwounded leaves (Fig. 8). Surprisingly, pretreatment of the detached flax leaves with 1 mM aspirin led to a reduction of the wound-induced accumulation of AOS transcripts. This reduction occurred in both the damaged and the systemic nondamaged tissues. Similar results were obtained by using SA (data not shown). These data strongly suggest that aspirin or SA inhibit wound-induced JA biosynthesis by blocking AOS gene expression at the transcriptional level.

DISCUSSION

AOS has been postulated to play a key role in the biosynthesis of JA (Vick and Zimmerman, 1987; Hamberg and Gardner, 1992). Overexpression of the flax AOS cDNA in transgenic potato plants leads to an increase in endogenous JA levels by 8- to 12-fold compared with nontransformed potato plants (Harms et al., 1995). These results suggest that potato leaves possess the factors needed for JA formation and that no previous stimuli are required for their release. Furthermore, they suggest that the amounts of JA present in normal plants are determined by the amount of AOS activity, which may represent the major rate-limiting step of the biosynthetic pathway of JA. AOS has been found to be ubiquitous in plants (Brash and Song, 1995;

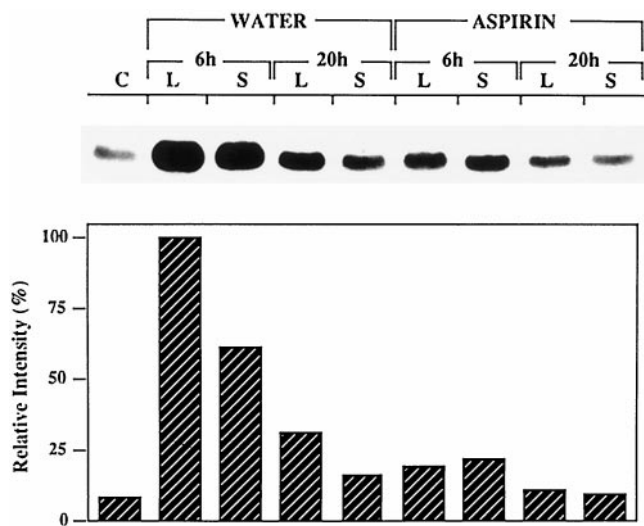


Figure 8. Influence of aspirin on AOS mRNA levels in flax leaves. Excised flax plants were preincubated with buffer or aspirin for 2 h, as described previously, and were subsequently wounded. RNA was isolated from flax leaves of intact plants before wounding (C) and from directly wounded (L) and systemic unwounded (S) leaves at the times shown after treatment. Amounts of RNA were confirmed as described in Figure 1. Blots were probed with the flax AOS cDNA and the corresponding densitogram is shown.

Simpson and Gardner, 1995) and differs in the distribution of its activity in different tissues (Vick and Zimmerman, 1987; Simpson and Gardner, 1995). Recently, Laudert et al. (1996) demonstrated that a single gene for AOS is likely present in the genome of *Arabidopsis*. Similar organization has been observed in flax, tomato, and potato plants (H. Peña-Cortés, unpublished data). AOS is constitutively expressed in most tissues of flax plants (Fig. 1), the amounts of transcript being higher in flax seeds. Its physiological role in this organ is unclear, because total enzyme activity is very high in seeds compared with vegetative tissues (Vick and Zimmerman, 1987). The constitutive AOS expression seen in all tissues may be required for the formation of AOS to maintain a certain amount of the protein required for the formation of basal levels of JA.

It is interesting that AOS gene expression can be enhanced transiently in flax leaves after mechanical wounding. This gene activation is not limited to the wounded leaves; it also takes place in the unwounded leaves located distally to the wounded leaves. The nondamaged leaves of a wounded plant readily accumulate AOS mRNA, albeit with a short delay compared with the directly wounded leaves, thus resulting in lower levels of AOS at a given time in the systemically induced leaves compared with the locally induced leaves. Elevation of AOS transcript amounts can be detected as soon as 10 min after wounding (data not shown), with the increase being clearly detectable after 1 h and a maximum being reached at 5 to 7 h (about 3-fold higher than the levels present in unwounded leaves). This increase declines to 70% of the maximum value after 11 h, returning to values similar to those of the nontreated leaves 26 h after treatment.

The kinetics of the wound-induced accumulation of flax AOS mRNA differ from the wound kinetics of *Arabidopsis* AOS (Laudert et al., 1996). Although *Arabidopsis* AOS transcripts also increase transiently after mechanical wounding, they reach maximum levels between 60 and 90 min and decrease rapidly in the next 30 min. As mentioned above, the increase of flax AOS transcript levels remains elevated for several hours, and a decline can only be observed 11 h after the start of the treatment. AOS mRNA accumulation in *Arabidopsis* leaves correlates with an increase in JA levels. This increase occurs in the first 90 min after wounding and levels decline rapidly to basal levels in the next 2 h (Laudert et al., 1996). JA accumulation in flax leaves follows different kinetics than in *Arabidopsis* leaves. The JA levels increase and reach maximum levels 6 h after wounding and remain elevated for the next 26 h. Both AOS gene expression and JA biosynthesis are affected in both *Arabidopsis* and flax plants after mechanical wounding.

However, there are clear differences between the genera in response to this stimuli. Whereas *Arabidopsis* plants react quickly by a transient accumulation of AOS mRNA and JA after wounding, the response in flax leaves occurs more slowly and the accumulation of AOS transcripts and JA remains detectable for several hours. Although such differences could be explained by dissimilarities in the procedure used to perform the mechanical wounding or in experimental conditions, fundamental differences in the metabolic pathway between both plants cannot be ruled out. For instance, the wound-induced accumulation of JA in flax resembles the kinetics observed in potato and tomato leaves, in which the maximum levels of JA are reached 6 h after mechanical wounding (Peña-Cortés et al., 1993, 1995; Harms et al., 1995; Herde et al., 1996). Furthermore, JA pools also increase transiently in tobacco leaves after mechanical damage (Baldwin et al., 1994). Within 0.5 and 2 h after damage, the shoot and the root JA pools of damaged tobacco plants were significantly greater than the corresponding levels of unwounded plants. JA levels of damaged leaves decreased to levels found in unwounded plants 10 h after harvest. Together, these results suggest that plants may respond to the same stimulus with some dissimilarities, which may depend on certain differences in their metabolic (biosynthetic and/or catabolic) pathways.

AOS involvement in JA biosynthesis has been well documented (Vick and Zimmerman, 1987; Song and Brash, 1991; Harms et al., 1995), as has the involvement of JA in the regulation of wound-induced gene expression in plants (Farmer, 1994; Peña-Cortés et al., 1995). JA has also been shown to be involved in systemic wound-induced gene expression (Farmer and Ryan, 1992; Peña-Cortés and Willmitzer, 1995; Bergey et al., 1996). Because AOS mRNA accumulates in directly wounded tissues and, based on the correlation with JA accumulation, an increase of JA in the systemic unwounded leaves as a result of AOS gene activation is also to be expected. The local and systemic AOS gene activation supports earlier results on the involvement of increased levels of JA in both local and systemic wound-induced gene activation (Bergey et al., 1996; Herde et al., 1996; Peña-Cortés et al., 1996).

Several components of the octadecanoid pathway, such as linolenic acid, 13-HPLA, and 12-oxo-PDA, as well as JA, are able to activate wound-responsive genes (Farmer 1994). Flax AOS gene expression is activated by treatment with JA. This activation occurs in a dose-dependent manner, with 50 μM being the most effective concentration. Similar results were obtained by applying the same concentration of 12-oxo-PDA and the bacterial phytotoxin coronatine (data not shown). Although coronatine exhibits a high structural similarity to 12-oxo-PDA, it cannot be converted to JA (Weiler et al., 1994). Thus, coronatine-induced AOS gene expression strongly supports previous results postulating the involvement of the intermediates of the JA biosynthetic pathway (i.e. 12-oxo-PDA) in the modulation of wound-responsive genes (Weiler et al., 1994; Bleichert et al., 1995). Furthermore, these results strongly suggest that the precursors of JA, 12-oxo-PDA, and JA itself are able to activate their own biosynthetic pathway in flax leaves.

As mentioned previously, wound-induced AOS mRNA accumulation take place very fast, the transcript being detectable in flax leaves within the first 10 min after wounding (data not shown) and after 15 min in Arabidopsis plants (Laudert et al., 1996). However, this fast activation can be blocked with protein-synthesis inhibitors. Cycloheximide (an inhibitor of cytoplasmic protein biosynthesis) prevents AOS mRNA accumulation upon wounding, whereas chloramphenicol (affecting protein biosynthesis in the chloroplasts) does not. These results suggest that the wound-induced activation of AOS gene expression depends on the *de novo* biosynthesis of cytoplasmic protein(s) that are required in the signaling-pathway-modulating wound response (Peña-Cortés et al., 1989).

Both SA and aspirin lead to a reduction in the wound-induced JA accumulation in flax leaves. These results confirm the previous finding by Peña-Cortés et al. (1993) that application of these substances inhibits the effect of wounding in JA accumulation. Evidence obtained by analyzing *pin2* gene expression using different intermediates of the JA biosynthetic pathway in the presence and absence of aspirin or SA showed that only 12-oxo-PDA and JA overcome the inhibitory effect of these substances. Thus, it was suggested that SA and aspirin may inhibit the enzymatic activity of AOS, the enzyme responsible for the conversion of 13-HPLA to 12-oxo-PDA. These substances are synthesized by a pathway involving oxygenation and cyclization steps, which shows a strong resemblance to the proposed biosynthetic pathway of eicosanoids in animals (Needleman et al., 1986). It has been shown that the chemical structures of JA and 12-oxo-PDA are very similar to those of animal prostaglandins (Vick and Zimmerman, 1983). In plants 12-oxo-PDA can be produced either by an enzymatic process involving the enzymes AOS and AOC or by spontaneous cyclization of the allene oxide, resulting in a racemic mixture of 12-oxo-PDA, one raceme being the direct precursor of (+)-7-iso-JA (Hamberg and Gardner, 1992). The allene oxide produced by the action of AOS can spontaneously be converted to 12-oxo-PDA by a mechanism that does not require the involvement of AOC. If AOC activity alone was affected by the inhibitors, we should be able to observe a small fraction of racemic 12-oxo-PDA as a

result of spontaneous formation and a corresponding accumulation of JA upon mechanical wounding, even in the presence of aspirin or SA. Because we could not detect any increase of JA after such treatments, we assume that both aspirin and SA act on AOS and not on AOC.

Aspirin and SA were postulated to prevent the formation of 12-oxo-PDA by negatively affecting AOS enzymatic activity (Peña-Cortés et al., 1993). In human cells aspirin acetylates a Ser residue of the catalytic site of prostaglandin endoperoxide synthetase, causing an irreversible inhibition of the cyclooxygenase activity and thus preventing prostaglandin biosynthesis (Van der Ouderdaa et al., 1980). Furthermore, SA is an effective inhibitor of cyclooxygenase-2 activity, thereby preventing the formation of prostanoids at the site of inflammation (Mitchell et al., 1997). Examination of flax AOS activity using a protein extract from flax seeds in the absence or presence of increasing concentrations of SA or aspirin clearly shows that neither substance influences the formation of 12-oxo-PDA. These data indicate that the components (either the enzymes AOS and AOC or via a spontaneous mechanism) allowing the formation of 12-oxo-PDA in this system are not affected by aspirin or SA. Both substances prevent the accumulation of AOS mRNA in flax leaves after mechanical wounding. Flax leaves pretreated with aspirin clearly show lower levels of AOS transcripts upon wounding than leaves of untreated plants. This effect is not limited to local AOS gene expression but is also seen in the systemic accumulation of AOS mRNA. These results, however, do not permit the determination of whether both substances alter the steady state of AOS transcript levels or the wound-induced accumulation of AOS mRNA. Because the AOS protein levels are not changed in the treated leaves (data not shown), the results obtained here may indicate that both SA and aspirin suppress the wound-induced accumulation of AOS, most likely by altering some component of the transcriptional machinery.

Several studies have demonstrated the role of SA and aspirin in the regulatory mechanisms involved in different biological processes. In HeLa cells, SA activates the human heat-shock transcription factor. Like heat shock, salicylate may interfere with protein synthesis or lead to the accumulation of aberrant newly synthesized proteins (Jurivich et al., 1992). It has also been reported that SA and aspirin inhibit the activation of the transcription of NF- κ B in transfected T cells (Kopp and Ghosh, 1994). Most of the genes known to be activated by NF- κ B are involved in the immune and inflammatory responses (Grilli et al., 1993). Bestatin, an inhibitor of some aminopeptidases in plants and animals, inhibits NF- κ B degradation in liver nuclei, whereas cycloheximide blocks the activation of NF- κ B (Cressman and Taub, 1994). More recently, Schaller et al. (1995) reported that Bestatin acts as a powerful inducer in tomato leaves of defense genes that are also induced by herbivore attack, mechanical wounding, systemin, and JA. Because Bestatin does not influence the internal levels of JA, it was assumed that the site of action of Bestatin is located downstream of the octadecanoid pathway. This substance may exert its effect at or near the levels of gene

transcription by inhibiting a regulatory protease (Schaller et al., 1995).

It has also been reported that aspirin and SA influence animal and plant gene expression by affecting the accumulation of the corresponding transcripts. Thus, low levels of aspirin and SA suppress the accumulation of the 2.7-kb prostaglandin synthase mRNA after interleukin-1 treatment (Wu et al., 1991). These substances also inhibit the wound-inducible ACC synthase transcript in tomato fruit (Li et al., 1992). Moreover, Kim et al. (1992) demonstrated that SA and aspirin inhibit the Suc response of the potato *pin2* promoter, suggesting the interaction of SA with a receptor. In addition, salicylate-like drugs have the ability to inhibit the accumulation of steady-state levels of nitric oxide synthase mRNA and subsequent nitric oxide synthase-2 enzymes and nitrite production in cultured neonatal rat cardiac fibroblasts (Farivar and Brecher, 1996; Farivar et al., 1996).

In summary, the similarity of the effects of aspirin, SA, and cycloheximide on NF- κ B in animal cells and on the wound-induced AOS gene expression in flax plants strongly suggests the presence of some common features between the signal transduction pathway mediating stress response in both animals and plants, as already proposed by Bergey et al. (1996).

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